Amino Acids (2012) 42:1151–1161
DOI 10.1007/s00726-010-0773-2

REVIEW ARTICLE

Advanced glycation endproducts: from precursors to RAGE: round and round we go

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Received: 17 July 2010 / Accepted: 1 September 2010 / Published online: 19 October 2010

Abstract The formation of advanced glycation endproducts (AGEs) occurs in diverse settings such as diabetes, aging, renal failure, inflammation and hypoxia. The chief cellular receptor for AGEs, RAGE, transduces the effects of AGEs via signal transduction, at least in part via processes requiring the RAGE cytoplasmic domain binding partner, diaphanous-1 or mDia1. Data suggest that RAGE perpetuates the inflammatory signals initiated by AGEs via multiple mechanisms. AGE–RAGE interaction stimulates generation of reactive oxygen species and inflammation—mechanisms which enhance AGE formation. Further, recent data in type 1 diabetic kidney reveal that deletion of RAGE prevents methylglyoxal accumulation, at least in part via RAGE-dependent regulation of glyoxalase-1, a major enzyme involved in methylglyoxal detoxification. Taken together, these considerations place RAGE in the center of biochemical and molecular stresses that characterize the complications of diabetes and chronic disease. Stopping RAGE-dependent signaling may hold the key to interrupting cycles of cellular perturbation and tissue damage in these disorders.

Keywords Glycation · Oxidative stress · Receptor for advanced glycation endproduct · Diabetes · Atherosclerosis · Hypoxia

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Advanced glycation endproducts and RAGE: where it began

The products of nonenzymatic glycation and oxidation of proteins, the advanced glycation endproducts (AGEs), form predominantly on lysine and arginine groups of proteins. Two major dicarbonyl compounds that are key precursors of AGE formation include 3-deoxyglucosone (3-DG) and methylglyoxal (MG) (Thornalley et al. 1999). In endogenous settings, AGEs form and accumulate in diabetes, aging, renal failure and inflammation (Monnier 2003). Furthermore, hypoxia triggers AGE formation in cultured endothelial cells and monocytes/macrophages (Chang et al. 2008; Xu et al. 2010). In diabetes, the formation of AGEs is accelerated due to high levels of blood glucose. In contrast, in innate aging in the non-diabetic state, AGE formation occurs much more slowly and particularly on long-lived proteins. Exogenously, preparation of food at high temperatures is reported to facilitate AGE formation—suggesting that total body AGE burden may reflect absorption from the gut as well as endogenous net formation versus clearance or detoxification (Koschinsky et al. 1997). AGEs have been identified in atherosclerotic plaques, even in the absence of diabetes, and AGE epitopes are found in in vitro prepared oxidized low density lipoprotein (oxLDL) (Harja et al. 2008; Zieman and Kass 2004). AGEs are heterogeneous substances—a prevalent AGE in vivo is carboxymethyl lysine (CML) AGE and is a form of non-fluorescent AGE (Thornalley 2003). Other AGEs such as pentosidine are characterized by their ability to form cross-links and to fluoresce (Thornalley 2003).

A key question in the biology of AGEs is whether these modified moieties are solely biomarkers of diseases in which they accumulate, or whether AGEs initiate signal transduction, thereby altering gene programs and cellular fate and function. Much evidence points to roles for AGEs

Table 1 AGEs and human disease: examples of measurement of AGE levels and relationship to disease

Source	Major finding	Reference
Plasma	↑ AGE pentosidine in renal failure	Friedlander et al. (1996)
Skin	↑ Skin Autofluorescence in systemic lupus Erythematosus vs. controls	Nienhuis et al. (2008)
Serum	↓ AGEs post-statin treatment In nonalcoholic steatohepatitis	Kimura et al. (2010)
Serum	↑ AGEs in type 1 diabetic patients with complications	He et al. (2001)
Serum	↑ AGEs in nondiabetic subjects with insulin resistance	Tahara et al. (2010)
Serum	↑ Pentosidine and CML AGEs in type 2 diabetic subjects with diabetic retinopathy	Ghanem et al. (2010)
Serum	↑ Pentosidine AGE levels in heart failure	Koyama et al. (2007)
Serum	↑ CML AGE levels predict mortality in hemodialysis subjects	Wagner et al. (2006)
Urine	↑ Pentosidine with disease activity in inflammatory bowel disease	Kato et al. (2008)
Vascular tissue	↑ AGE with aging, diabetes	Halushka et al. (2009)

in both situations. A substantial literature exists describing associations between plasma, serum, urine or skin (as examples) levels of AGEs with disease states from diabetes to inflammation in animal models and in human subjects. In some settings, such levels of AGEs correlate with disease and/or its severity. Table 1 contains examples of studies in which measurement of AGEs reflected disease status. Furthermore, in serial tissue samples retrieved from subjects enrolled in the type 1 diabetes control and complications trial (DCCT), skin biopsies were used to identify AGEs that may be predictors of the development of diabetic complications such as retinopathy and nephropathy (Genuth et al. 2005; Monnier et al. 1999).

In addition to their ability to mark disease patterns, AGEs may alter properties of collagens and elastins, and, thereby vascular stiffness or the composition and function of basement membranes, such as in the eye or the kidney. The AGE cross link breaker—alagebrium or ALT-711, was found to reduce vascular stiffness in animal models and in human subjects of aging (Bakris et al. 2004). Our group hypothesized that one mechanism by which AGEs contributed to vascular stress was via interaction with cell surface receptors. Serial chromatography and radioligand binding assays were employed to screen bovine lung extracts (rich in endothelial cells, a cell type known to bind AGEs) for AGE binding activities. From such a strategy, a receptor for AGEs (RAGE) was purified to homogeneity (Neeper et al. 1992). RAGE remains among the best-characterized receptors for AGEs.

RAGE: a receptor for AGEs and distinct ligands

AGEs prepared in vitro by incubation of proteins with reducing sugars or those isolated from in vivo sources bind RAGE in a dose-dependent and saturable manner, $K_d \approx 50$ nM (Neeper et al. 1992). RAGE is expressed in multiple cell types at very low levels in the absence of disease with increased expression noted in a range of cell types and tissues in disease states, such as diabetes, neurodegenerative disorders, and autoimmune/inflammatory conditions. It is important to note that other receptors for AGEs have been described, some of which have been characterized as scavenger receptors (Lu et al. 2004). In contrast, RAGE is a member of the immunoglobulin superfamily. The molecular cloning of RAGE predicted that is composed of an extracellular domain of three immunoglobulin (Ig)-like domains—one V (variable) type Ig domain followed by two C (constant) type Ig domains. There is a single transmembrane spanning domain and a very short (<50 amino acids) cytoplasmic domain that is highly charged (Neeper et al. 1992). The cytoplasmic domain of RAGE is actively required for RAGE-dependent signaling, as indicated by experiments in which this domain of the receptor is deleted. AGE–RAGE bindings stimulates activation of diverse signaling cascades—from members of the Rho GTPase family, mitogen-activated protein kinases, phosphoinositol-3 kinase/Akt to Jak/stat pathways, as examples (Yan et al. 2010).

Our laboratory has recently shown that the cytoplasmic domain of RAGE, previously shown to be bind directly to extracellular-regulated kinase (erk), binds to a member of the formin family of cellular effector molecules, the FH1 domain of mDia-1 (diaphanous1; Hudson et al. 2008; Ishihara et al. 2003). Studies using small inhibitory RNAs (siRNA) and macrophages retrieved from mDia-1 null mice reveal that signal transduction initiated by RAGE ligands is markedly reduced compared to that observed in wild-type cells (Xu et al. 2010). Whether all ligand-stimulated RAGE signaling requires mDia-1 is yet to be

Table 2 RAGE ligands and putative domain(s) of interaction

Ligand	Domain(s) of interaction	Reference(s)
AGEs	V domain	Xie et al. (2008)
HMGB1	V domain	Hori et al. (1995)
S100B	V domain	Ostendorp et al. (2007), Dattilo et al. (2007)
S100A12	V, C1 domains	Xie et al. (2007)
Amyloid-β oligomers	V domain	Sturchler et al. (2008)
Amyloid-β aggregates	C1 domain	Sturchler et al. (2008)
S100A6	V, C2 domains	Leclerc et al. (2009)

discovered; it is plausible that distinct cell types or conditions do not require this molecule for RAGE ligand signaling.

In addition to AGEs, RAGE is a signal transduction receptor for non-AGE ligands. Hypochlorite oxidized albumin, a method used to prepare advanced oxidation protein products (AOPPs) bind RAGE and initiate signal transduction (Guo et al. 2008). Other RAGE ligand families include at least certain members of the S100/calgranulin family (such as S100B, S100A12, S100A4, S100A6, S100A8/9 and S100P), high mobility group box 1 (HMGB1), amyloid-β peptide and β-sheet fibrils, and mac-1 (Leclerc et al. 2009). Based on the seemingly diverse ligands that bind RAGE, the receptor has been added to the list of "pattern recognition receptors." In fact, most structural biology reports of RAGE indicate that a major site of diverse ligand binding is the first V-type Ig domain. Although some of the ligands appear to bind to the C1 or C2 domains, much evidence suggests that the V-domain is a major site of ligand engagement—pharmacological strategies to target RAGE have therefore focused in great part on this domain. Table 2 lists examples of RAGE ligands and the putative domain(s) of the receptor to which they bind.

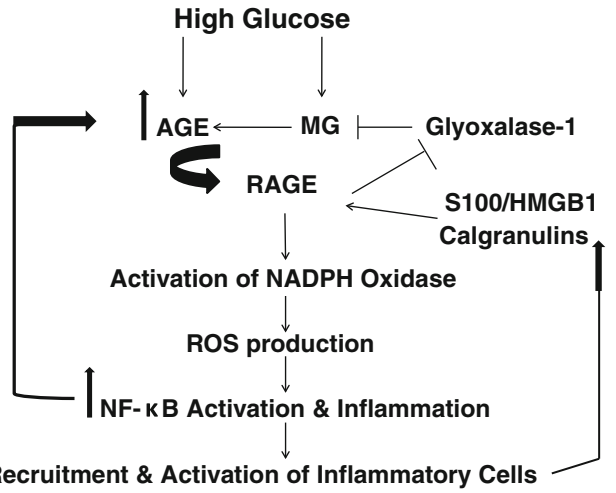

Fig. 1 AGE–RAGE and perpetuation of cellular stress in chronic disease. We hypothesize that settings such as hyperglycemia result in the production of AGEs. AGE interaction with the chief cell surface receptor has multiple cellular effects, including the generation of reactive oxygen species (ROS) via NADPH oxidase, and induction of cellular signaling. Sequelae include activation of inflammatory signals, at least in part via activation of NF-kB, leading to recruitment and activation of S100/calgranulin- and HMGB1-bearing inflammatory cells. Release of these molecules in foci of AGE accumulation perpetuates RAGE-dependent signaling, ROS production, activation of NF-kB and alterations in genomic profiles in target cells and tissues. Recent data indicate that RAGE action attenuates glyoxalase 1, as RAGE-deficient type 1 diabetic kidney reveals markedly lower methylglyoxal levels versus diabetic controls. Such considerations compound the RAGE-dependent cycles of AGE production and accumulation and participate, we propose, in the pathogenesis of diabetic complications and other chronic diseases

RAGE and its influence on ligand production and clearance

Early studies in diabetic RAGE deficient kidney suggested that AGE levels were reduced compared to levels observed in RAGE-expressing kidney tissue—with equal degrees of hyperglycemia (Myint et al. 2006). This finding suggested that RAGE might regulate levels of ligand—such that RAGE ligand activation of RAGE perpetuated cellular stress by sustaining production and/or impairing clearance of AGEs or their precursors. In fact, evidence for both scenarios has been published and provides a compelling story—among the many actions of RAGE is ensuring its own state of activation and ligand production.

Oxidative stress and inflammation are two potential mechanisms by which AGEs may be generated. It has been shown that myeloperoxidase plays a key role in generation of CML, suggesting that these stresses are triggers to production of the RAGE ligands (Anderson et al. 1999). RAGE has been linked to activation of NADPH oxidase and generation of reactive oxygen species (ROS; Wautier et al. 2001). Hence, AGE–RAGE interaction fuels production of ROS and signaling mechanisms, such as activation of NF-kB, which begets further inflammation and an evolving cycle of AGE production. Of note, recruitment of inflammatory cells to sites of AGE deposition may result in release of non-AGE RAGE ligands such as members of the

S100/calgranulin family and HMGB1, thereby promoting RAGE activation by distinct means (Fig. 1).

Recent data suggest the intriguing finding that RAGE regulates AGE detoxifying mechanisms, specifically mRNA and protein levels of glyoxalase 1 (glo1). Glo1 detoxifies methylglyoxal (MG), thereby depleting a major pool of AGE precursors (Thornalley et al. 2003). We reported that when type 1 diabetic OVE26 were bred into the RAGE null background, in parallel with marked reduction in pathological and functional endpoints of glomerulosclerosis and the preservation of glomerular filtration rate (GFR), levels of MG were much lower in diabetic OVE26 RAGE null mice versus OVE26 mice (Reiniger et al. 2010). Indeed, levels of MG in the diabetic OVE26 RAGE null mouse cortex were similar to those found in non-diabetic FVB cortex. Importantly, RAGE deficiency had no impact on levels of glucose or glycated hemoglobin; hence, the reduction in MG was not accounted for by reduced glucose. Rather, our data revealed that levels of Glo1 mRNA and protein, as determined by real time quantitative PCR and Western blotting, respectively, were significantly higher in OVE26 RAGE null versus OVE26 cortex (Reiniger et al. 2010). It should be noted that Glo1 is a glutathione-dependent enzyme (Thornalley et al. 2003). Therefore, an additional potential means by which RAGE deletion promoted lower levels of MG in the type 1 diabetic kidney cortex was via reduction in oxidative stress and consequent prevention of glutathione depletion (Fig. 1).

Taken together, these considerations suggest that RAGE plays multiple roles in executing the signal transduction mechanisms initiated by ligand binding, and that RAGE's actions contribute to perpetuation of AGE and pro-inflammatory ligand production, in part via facilitating a microenvironment conducive for ligand production (oxidative stress and inflammation) and by suppressing protective mechanisms, that is reduction in transcripts and mRNA of Glo1.

In the sections to follow, we review two settings in which AGEs form and accumulate and signal tissue stress through RAGE—diabetes and hypoxia.

RAGE and the complications of diabetes

Two of the best-characterized settings in which RAGE has been studied in diabetic complications have been in macrovascular complications and in diabetic nephropathy. Both are two major sequelae of diabetes that result in significant morbidity and mortality. Diabetes significantly accelerates the development of heart attacks and strokes and is a leading cause of renal failure (van Dieren et al. 2010). Ironically, a major sequelae of end-stage renal disease is marked acceleration of atherosclerosis (Saran and DuBose 2008). In both macrovascular tissues and in renal failure, increases in the AGE–RAGE axis have been demonstrated in animal models and in human subjects.

Multiple experimental tools have been developed to probe the RAGE axis in disease settings. For example, the soluble extracellular domain of RAGE has been prepared from an insect cell expression system and acts as a ligand decoy (Wautier et al. 1996). Others have prepared blocking antibodies to RAGE which may be administered chronically in animal models (DeVriese et al. 2003). Genetic deletion of RAGE has been accomplished in mice and these animals have served as a key test of the RAGE hypothesis.

RAGE and atherosclerosis

Studies in human subjects have shown that RAGE is expressed in atherosclerotic plaques in both diabetic and non-diabetic subjects and suggest that peripheral blood mononuclear cells (PBMCs) in vulnerable subjects may bear the RAGE axis signature as well. Mahajan and colleagues performed a study of non-diabetic subjects with angiogram-positive or negative evidence of atherosclerosis. PBMCs retrieved from subjects with pre-mature coronary artery disease revealed increased expression of RAGE and S100A12, and there was a positive correlation with high sensitivity C-reactive peptide (hsCRP) levels and a negative correlation with circulating levels of soluble RAGE (Mahajan et al. 2009). Further, the degree of coronary artery disease correlated positively with the extent of RAGE and S100A12 expression. In diabetic subjects, Burke and colleagues examined atherosclerotic plaques from diabetic and non-diabetic subjects and observed that although RAGE and S100A were expressed in both groups of subjects' plaques, the extent of expression was significantly higher in the diabetic versus the non-diabetic populations. Diabetic plaques exhibited more macrophages compared with non-diabetic plaques and this correlated with RAGE and S100A12 expression (Burke et al. 2004).

An evolving area in the study of RAGE and human subjects is the measurement of circulating levels of soluble RAGE. In human subjects, there are two distinct mechanisms and "forms" of soluble RAGE. The first is "total" soluble RAGE which encompasses the material cleaved from cell surface receptor. Putative mechanisms for release of these forms of soluble RAGE include the actions of ADAMs (Raucci et al. 2008). Yonekura and colleagues described a second form of "endogenous secretory" form of soluble RAGE in which a naturally occurring splice variant is responsible for production of a distinct form of soluble receptor (Yonekura et al. 2003). Based on the sequence, endogenous secretory RAGE possesses novel

Table 3 Human studies assessing "soluble RAGE" levels: examples of studies in cardiovascular disease

Soluble RAGE form	Results	Reference
"Total" soluble RAGE	↓ Soluble RAGE levels in non-diabetic Italian men with vs. without coronary artery disease	Falcone et al. (2005)
"Total" soluble RAGE	↓ Soluble RAGE levels in subjects with angiographically proven coronary artery disease vs. controls with symptoms but negative angiography (non-diabetic)	Mahajan et al. (2009)
esRAGE (splice variant)	↑ esRAGE levels in type 2 diabetic Japanese men correlated with higher levels of HDL	Katakami et al. (2006)
esRAGE (splice variant)	↓ esRAGE levels in subjects with metabolic syndromes and carotid or femoral atherosclerosis	Koyama et al. (2005)
esRAGE (splice variant)	↓ esRAGE levels associated with carotid intima-media thickness in type 2 diabetic subjects	Katakami et al. (2007)
"total" soluble RAGE	Soluble RAGE levels ↑ with advancing New York Heart Association functional status	Koyama et al. (2008)

amino acids within the region of the C2-type Ig domain that confers a unique epitope for antibody and ELISA development. Table 3 lists examples of human studies in which levels of soluble RAGEs were tested for associations with cardiovascular disease. The conclusion, at this point, is that there is no conclusive evidence that either form of soluble RAGE and/or the level correlate with increased propensity to or protection from atherosclerosis. One possible reason for the discrepancies is that studies do not consistently report both forms of soluble RAGE—perhaps it is the ratio between the two forms of RAGE that is more predictive of cardiac disease rather than absolute levels of either form. What is certain, however, is that multiple studies have now shown that treatment with agents such as statins or perindopril increased soluble RAGE levels in human subjects (Forbes et al. 2005; Santilli et al. 2007). In order to attempt to definitively establish levels of soluble RAGE as a biomarker of disease status or severity, future studies must examine larger subject populations with concomitant measurement of both "total" soluble RAGE and endogenous secretory soluble RAGE.

Studies in animal models have used two distinct approaches to date to assess the role of RAGE in diabetic atherosclerosis. In the first set of studies, soluble RAGE was administered to mice susceptible to the development of accelerated atherosclerosis in diabetes. Most of the published studies to date have employed the apolipoprotein E deficient mice in which atherosclerosis develops spontaneously on a normal chow diet. In those studies there were two principal findings: first, induction of diabetes by chemical approaches (streptozotocin) or by breeding into the type 2 diabetic db/db background accelerated atherosclerosis (Bucciarelli et al. 2002; Park et al. 1998; Wendt et al. 2006). As in human subjects, there was increased macrophage density/plaque area in diabetes versus age-matched non-diabetic cohorts. When soluble RAGE was administered to the diabetic mice, it arrested the vascular inflammation to a highly significant degree. In parallel, atherosclerotic lesion area, number and degree of complexity were highly reduced in animals receiving soluble RAGE versus vehicle. Of note, soluble RAGE had no impact on the degree of hyperglycemia or the lipid number and profile. Total levels of cholesterol and triglyceride were not different between soluble RAGE versus vehicle-treated diabetic mice.

In other studies, genetic modification of RAGE was employed. Two different approaches were used. In the first, homozygous RAGE null mice were bred into the apoE null background (both diabetic and non-diabetic state) or into the low density lipoprotein receptor (LDLR) background (non-diabetic state; Bu et al. 2010; Harja et al. 2008; Soro-Paavonen et al. 2008; Sun et al. 2009). In these experiments, deletion of RAGE resulted in marked reduction in atherosclerotic lesion area and vascular inflammation. In another study, transgenic mice expressing cytoplasmic domain-deleted mice (driven by the pre-proendothelin 1 promoter) when bred into the apoE null background displayed much less atherosclerosis than their apoE null controls (Harja et al. 2008). In all cases, RAGE deletion had no effect on levels of glucose or lipids, suggesting that the impact of RAGE modification of expression or function had no effect on distinct risk factors that have been shown to contribute to atherosclerosis.

Bu and colleagues performed affymetrix genomic arrays on aortas of diabetic and non-diabetic apoE null mice at 9 weeks of age; the premise of the study was to identify vascular factors that predispose to atherosclerosis and not the lesion gene expression profiles themselves (Bu et al. 2010). A chief pathway found to be impacted by diabetes and by RAGE was the ROCK1 branch of the transforming growth factor-β (Tgf-β) signaling pathway. Further, the chief cell type expressing these molecules in diabetic and

RAGE-expressing apoE null aorta was the smooth muscle cell. An extensive series of studies using cultured primary murine aortic smooth muscle cells expressing or devoid of RAGE traced RAGE ligand dependent smooth muscle cell properties to ROCK1. Using two pharmacological antagonists of ROCK1, it was shown that RAGE ligand S100B-mediated smooth muscle cell proliferation and migration were significantly reduced (Bu et al. 2010).

Ongoing studies are probing the role of RAGE in later stages of atherosclerosis using mice with established lesions.

RAGE and diabetic kidney

Multiple pharmacological approaches have been employed to test the role of RAGE in animal models of diabetes-associated nephropathy. Administration of soluble RAGE to genetically type 2 diabetic db/db mice resulted in reduction in distinct facets of diabetic kidney disease. Glomerular and mesangial areas were significantly reduced, as was glomeular basement membrane thickening. Albuminuria was reduced in this model as well (Wendt et al. 2003). Others used novel anti-RAGE antibodies to show that key indices of nephropathy were reduced after long-term treatment in mouse models of both types 1 and 2 diabetes (Flyvbjerg et al. 2004; Jensen et al. 2006).

Genetic studies have yielded important insights into roles for RAGE in diabetes-associated kidney disease. In a study employing a transgenic mouse approach, overexpression of megsin and RAGE in transgenic mice resulted in marked glomerular hypertrophy, diffuse mesangial expansion, inflammatory cell infiltration, and interstitial fibrosis compared with transgenic mice without overexpression of RAGE (Inagi et al. 2006). In two studies, Wendt and colleagues and Myint and colleagues showed that mice devoid of RAGE displayed significant protection against early indices of nephropathic changes. In these studies, significant reduction in genes associated with matrix expansion was noted in RAGE deficient versus wild-type mice (Myint et al. 2006; Wendt et al. 2003). These findings underscore an intriguingly similar pattern of RAGE-dependent gene expression in the diabetic kidney, as well as in the diabetic aorta (Bu et al. 2010). Ongoing studies are probing the relationship between RAGE, ROCK and Tgf-β signaling in the macro- and microvasculature in diabetes.

Despite the compelling suggestion from this work that RAGE contributed to nephropathic changes in the diabetic kidney, pitfalls of the available models included the lack of highly advanced kidney complications typical of long-term human diabetes. To address this issue, the Animal Models of Diabetic Complications Consortium (AMDCC) was formed and this organization has focused on development of more advanced models of diabetes associated changes in the kidney. The OVE26 mouse developed by Epstein and colleagues is a model of type 1 diabetes driven by excess transgenic calmodulin in the pancreatic islets (driven the rat insulin promoter). As reported in the literature and replicated in our laboratory, extensive albuminuria, glomerular sclerosis, podocyte effacement, thickening of the GBM and frank loss of GFR (approximately 30% at age 7 months) occurs in OVE26 mice (Zheng et al. 2004). To definitively test the role of RAGE, we bred OVE26 mice (FVB genetic background) into the RAGE null background (backcrossed >10 generations into FVB). Highly significant reductions in % glomerular sclerosis, podocyte foot process effacement, and albuminuria were observed. By insulin clearance studies, a nearly 30% reduction in GFR was noted in OVE26 mice versus FVB controls at age 7 months (Reiniger et al. 2010). In marked contrast, OVe26 RAE null mice displayed no decrement in GFR. GFR in OVE26 RAGE null mice was identical to that in FVB mice without diabetes. Notably, glucose and glycated hemoglobin levels were not altered by deletion of RAGE, thus, identical degrees of hyperglycemia were capable of driving kidney pathology in the diabetic mice devoid of RAGE.

To probe underlying mechanisms, affymetrix genomic arrays were performed in isolated glomeruli immediately after the first demonstration of albuminuria in OVE26 mice. Diabetic versus non-diabetic glomeruli revealed a marked increase in mRNA transcripts encoding Tgf-β, TgF-β induced and Alpha(1)-4 collagen. In OVE26 RAGE null mice, transcript levels of these key pro-fibrotic factors were much lower than those observed in wild-type mice (Reiniger et al. 2010). Taken together, these data showed for the first time that RAGE deletion prevented loss of GFR in this mouse model and hold promise for clinical studies in which preservation of glomerular function will be a defining endpoint.

In the section to follow, we will discuss a distinct setting in which AGEs form, hypoxia, and consider the role of RAGE in transducing AGE signals to the target tissue.

Hypoxia: generating AGEs and interaction with RAGE

Previous studies revealed that exposure of endothelial cells to in vitro-applied hypoxia stimulated rapid generation of AGE-reactive epitopes into cellular supernatants. By 10–15 min exposure to acute hypoxia, anti-AGE antibodies detected AGEs in cellular supernatants. A key consequence of hypoxia is the rapid upregulation of early growth response-1 (Egr-1), a transcription factor implicated in tissue damage, at least in part via transcriptional regulation of inflammatory and prothrombotic genes—mechanisms which cause tissue damage in the hypoxic setting. Both mRNA transcripts and protein levels of Egr-1 were higher

in hypoxia- versus normoxia-exposed murine and human endothelial cells, and activity of Egr-1 was increased, as demonstrated by electrophoretic mobility shift assays (Chang et al. 2008). As hypoxia stimulated generation of AGEs, we tested the hypothesis that RAGE contributed to hypoxia-stimulated AGE signaling. Experiments using RAGE deficient primary murine aortic endothelial cells or human aortic endothelial cells transfected with siRNAs to reduce RAGE expression revealed striking attenuation of hypoxia-stimulated upregulation of Egr-1. In vivo, compared to wild-type mice, RAGE deficient mice displayed a highly significant decrease in Egr-1 transcription and activity compared to wild-type mice. Consistent with a role for AGEs, pretreatment of the animals with aminoguanidine, an AGE inhibitor, suppressed hypoxia-stimulated upregulation of Egr-1 (Chang et al. 2008). One caveat in the interpretation of these studies is that the beneficial effects of aminoguanidine might have been related to non-AGE lowering effects. It has been suggested that aminoguanidine has anti-oxidant properties. Finally, it is important to note that the endothelial cell supernatants were assessed for production/release of S100/calgranulin epitopes and HMGB1. Even after multi-fold concentration of the supernatant material, there was no evidence that hypoxia released these molecules within the time course preceding upregulation of Egr-1 expression (Chang et al. 2008).

These concepts have recently been tested in monocytes/macrophages to address if hypoxia generates AGEs in these cells, and if so, the potential relationship to RAGE. Analogous to endothelial cells, macrophages released AGE epitopes within minutes of exposure to hypoxia. In contrast, there was no evidence of release of S100/calgranulins or HMGB1 under these conditions. Deletion of RAGE or reduction of RAGE expression using siRNA techniques resulted in suppression of hypoxia-stimulated regulation of Egr-1 in both murine and human macrophages (Xu et al. 2010). In that work, we extended our findings for the first time to test the premise that mDia-1 was required for the effects of AGEs in hypoxia. Using mDia-1 deficient macrophages and macrophages with reduced mDia-1 expression (siRNA), a marked reduction in hypoxia-stimulated upregulation of Egr-1 was observed (Xu et al. 2010).

These observations were then tested in animal models of cardiac ischemia and reperfusion injury. First, in the isolated perfused heart, induction of ischemia/reperfusion resulted in rapid production of MG in the heart that was prevented by soluble RAGE (Bucciarelli et al. 2006). Furthermore, in a distinct system, a murine model of left anterior descending coronary artery occlusion and reperfusion, it was found that even in the absence of diabetes, rapid production of methylglyoxal occurred in the heart tissue during the ischemic phase (Aleshin et al. 2008). Interestingly, AGE epitopes were detectable in the cardiac tissue somewhat later in the course of the experiments, during the reperfusion phase, perhaps reflecting that MG is an AGE precursor. In those studies, deletion of RAGE resulted in much smaller myocardial infarcts under these conditions compared to wild-type mice, in parallel with reduced loss of cardiac function as detected by echocardiography (Aleshin et al. 2008).

MG has also been implicated in other organ systems besides the heart, such as in the kidney. In the kidney, particularly in the tubulo-interstitium, ischemia results in reduced glyoxalase activity and hence an increase in MG in the damaged renal tubular cells (Kumagai et al. 2009). Interestingly, this could be prevented by overexpression of glyoxalase 1 in vivo in rats. Overexpression of human glyoxalase 1 resulted in reduced ischemia/reperfusion injury in the kidney, as demonstrated by functional and histological endpoints. In that study, the authors measured levels of carboxyethyl lysine (CEL), an AGE formed via MG and found that its accumulation was lower in the transgenic rats versus the wild-type control animals. Furthermore, in parallel with decreased MG and CEL AGE, reduced oxidative stress and tubular cell apoptosis were observed. In in vitro studies, siRNA knockdown of glyoxalase 1 exacerbated cell death induced by hypoxia/reoxgenation in rat tubular cells (Kumagai et al. 2009).

Taken together, these data suggest that AGE–RAGE-mDia-1 is a novel axis for transducing the effects of hypoxia on tissue damage at least in endothelial cells and macrophages. In the section to follow, we link the MG–AGE–RAGE pathway to the polyol pathway enzyme, aldose reductase (AR).

Aldose reductase, methyglyoxal and AGE–RAGE: a vicious cycle of cell stress

As discussed earlier, aging is also associated with increased accumulation of AGEs. In Fischer 344 rats, aged vascular tissue displayed increased expression and activity of AR and increased expression of RAGE compared to young rats (McCormick Hallam et al. 2010). Furthermore, increased vascular accumulation of MG was observed in aged vasculature, which was prevented by administration of the AR inhibitor, zopolrestat, to aged animals. In parallel, aging-associated endothelial dysfunction, as measured in aortic ring responses to acetylcholine, were impaired in aged Fischer 344 rat vascular rings versus rings retrieved from young rats. Treatment with zopolerstat reversed aging-associated endothelial dysfunction. Furthermore, consistent with the premise that AR-mediated MG/AGE production signaling endothelial dysfunction at least in part via RAGE, the effects of soluble RAGE were tested in aged rats. Administration of soluble RAGE to aged rats also

prevented aging-associated impairment in endothelial function, thereby linking AR to AGE–RAGE–mediated endothelial dysfunction of aging (McCormick Hallam et al. 2010).

Notably, extensive data link AR to ischemia/reperfusion induced tissue injury, thereby suggesting that at least one key means by which AR exerts its maladaptive effects in diverse settings is via triggering production of the AGE precursor MG in diverse settings such as ischemia/reperfusion, diabetes and aging. Once formed, AGEs have multiple effects in the tissues, and via RAGE, stimulate cellular signaling and tissue damaging pathways. Interestingly, one consequence of AGEs is further increases in AR activity. In transgenic human AR versus wild-type smooth muscle cells, incubation with AGE-BSA resulted in even further increases in AR activity; these changes were attenuated by ARI treatment (zopolrestat) (Dan et al. 2004). Further, increased AGE–AR pathway was associated with higher expression of inflammatory mediators in these cells (intercellular adhesion molecule-1, monocyte chemoattractant protein-1, and activation of protein kinase C). Each of these increases was suppressed either by ARI or by introduction of AR antisense oligonucleotides (Dan et al. 2004).

Summary and perspectives

Taken together, the biologies of diabetes, aging and ischemia/reperfusion posse in common activation of the AR–MG–AGE–RAGE axis. Recent insights extend at least part of this axis (given current knowledge) to an intracellular signal transduction mediator, mDia-1. Linking these pathways or their component parts to susceptibility to these diseases or the extent of damage in the disease settings may be important for the design of personalized therapies for affected subjects. In this context, polymorphisms of AR and RAGE have been described, as have been genetic variants in the gene encoding glyoxalase 1 (Demaine 2003; Gale and Grant 2004; Hudson et al. 1998). To our knowledge, genetic variants in mDia-1 have not yet been described. In our model of this axis, however, it is plausible that altered degrees of RAGE signaling induced by ligand may differentially impact changes in cellular function. Together with measurements of MG and AGEs, measurement of soluble forms of RAGE may be useful as predictors of disease status.

In-depth studies in human subjects are indicated to establish the potential benefits of antagonizing this pathway in diabetes, aging, ischemia/reperfusion, inflammation and renal failure. As described above, the AR–MG–AGE–RAGe–mDia-1 axis is complicated by the concept that RAGE acts in both initiation and amplification of cellular perturbation—at east in part via regulation of ligand production, detoxification, accumulation and induced signal transduction in a diverse array of cell types. These considerations suggest that the development of algorithms to quantify both expression and activities of AGE production and signal transduction mechanisms might be most useful to establish subjects for whom modulation of this axis will be beneficial.

Acknowledgments This work was supported by grants from the US Public Health Service and the Juvenile Diabetes Research Foundation. The authors are grateful for the assistance of Ms. Latoya Woods in the preparation of this manuscript.

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